

Limited Value of Assays Using Detection of Immunoglobulin G Antibodies to the Two Recombinant Dense Granule Antigens, GRA1 and GRA6 Nt of *Toxoplasma gondii*, for Distinguishing between Acute and Chronic Infections in Pregnant Women

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An enzyme-linked immunosorbent assay (ELISA) using two recombinant antigens of *Toxoplasma gondii* (GRA1 and GRA6 Nt) was developed in order to differentiate between pregnant women with a serological profile of recently acquired infection and those with chronic infection. Both proteins were expressed in *Escherichia coli* as glutathione S-transferase fusion proteins. Thirty-two serum samples from subjects who presented seroconversion within 3 months before sampling (group 1; acute profile), 46 serum samples from women who had a positive serology at least 1 year before sampling (group 2; chronic profile), and 100 serum samples from pregnant women who were not infected by *T. gondii* (group 3) were examined for immunoglobulin G (IgG) reactivity. For both antigens, the specificity reached 98%. In both groups of infected patients, the overall sensitivity scored was 60% for GRA1 and 83% for GRA6 Nt. In group 1, 34% of sera reacted with GRA1 whereas 84% of sera reacted with GRA6 Nt; in group 2, however, sensitivities were 78.2 and 82.6%, respectively. Combination of the readings obtained with both antigens yielded a sensitivity of 91%. A serological follow-up of 10 women who seroconverted during pregnancy displayed three different serological patterns: (i) a GRA profile paralleling the IgG curve, as detected by the commercial kit, (ii) a GRA1 profile, or (iii) GRA1 and GRA6 Nt profiles remaining negative for at least 8 weeks after the reference test gave positive results. Taken together, these results suggest that neither GRA1 nor GRA6 Nt is sensitive enough to be used routinely to differentiate between acute and chronic toxoplasmic infections.

Maternal infection by *Toxoplasma gondii* during pregnancy may result in a fetal infection with consequences ranging from severe neurological abnormalities and chorioretinitis to sub-clinical infection at birth, which is marked by a risk of late onset of ocular lesions (19). The risk of transmission to the fetus and the severity of congenital infection depend on when, during pregnancy, the woman becomes infected: the probability of fetal infection varies from 6% at the end of the first trimester to 72% at 36 weeks of gestation (8). The severity of infection is greater in fetuses infected early in pregnancy than in those contaminated later (8). Because only 5% of women who seroconvert present clinical signs, diagnosis of maternal infection relies on serological tests. For pregnant women, these should not only detect seroconversion but also estimate the date of maternal contamination in order to estimate the risk of mother-to-child transmission and the severity of fetal infection. It is of the utmost importance to differentiate between recently acquired infections which probably occurred after conception and those acquired in the distant past with no risk to the fetus.

Serological tests available for the diagnosis of toxoplasmosis require antigens that are isolated either from peritoneal fluid

of acutely infected mice or from cultures. Production of large quantities of antigen can be limited by difficulties in standardization, which lead to batch-to-batch differences. Moreover, parasitic antigens used for serodiagnosis can be contaminated by host proteins. Recombinant antigens offer the possibility of developing standardized reagents and reducing their production costs. Use of the enzyme-linked immunosorbent assay (ELISA) with *T. gondii* recombinant proteins has already been reported for the detection of immunoglobulin G (IgG) by using a single antigen (9, 17, 18, 20–22) or a panel of peptides (1, 10, 14). Detection of specific IgM by using this type of reagent has already been reported (1, 16, 21). Results are not homogeneous, and none of the recombinant proteins tested is currently used routinely. Among the polypeptides already tested, GRA1 and GRA6 Nt (corresponding to the hydrophilic N-terminal region of GRA6), expressed by the tachyzoite and bradyzoite stages of the parasite, have been shown to react with human sera, and combined results yielded an overall sensitivity of 98% (12). In addition, GRA1 has been reported to be a good marker of chronic toxoplasmosis (4), and GRA6 has been reported to be a good marker of acute infection (20). Other recombinant proteins were investigated in order to distinguish between the two phases of infection (20–22). Results are difficult to compare, because the criteria for classification of the two phases of the disease differ among publications.

In this study, we investigated the abilities of the two recom-

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binant proteins, GRA1 and GRA6 Nt, to detect specific IgG in sera of pregnant women with well-documented serological status in order to distinguish between the acute and chronic phases of toxoplasmosis.

MATERIALS AND METHODS

Serum samples. Serum samples were obtained retrospectively from patients during routine screening for toxoplasmosis at the laboratory of the Croix-Rousse Hospital, Lyon, France. Samples were tested for the presence of *Toxoplasma*-specific IgG and IgM by using an ELISA (Enzygnost Toxoplasmosis; Dade Behring, Marburg, Germany) with a lysate of the parasite as the antigen. This test is referred to below as the routine ELISA. The positive threshold determined by the manufacturer was 10 IU. Additional tests, such as detection of both IgG and IgM by flow cytometry (5) and detection of IgM by an immunocapture assay (ISAGA; BioMerieux, Marcy l'Etoile, France), were performed to investigate the serological pattern. Serum samples were divided into three groups according to serological results and the presence of a previous negative or positive sample. Group 1 comprised 32 serum samples drawn less than 3 months after seroconversion (acute infection). All group 1 patients had positive serology within the 3 months before the sample was taken. Group 2 comprised 46 positive serum samples with infection acquired in the distant past (chronic profile). All group 2 patients already had positive serology 1 year before the sample was taken. Group 3 comprised 100 serum samples from women who were negative for *T. gondii* IgG and IgM.

Seroconversion follow-up. Fifty-three serum samples from 10 women with documented seroconversion were referred to our laboratory at the request of obstetricians or general practitioners to estimate the age of the fetus when maternal infection occurred and to follow up patient serology. Patient follow-up ranged from 8 to 27 weeks after the first positive serum sample was detected by routine ELISA.

Recombinant antigens. Methods for the construction of pGEX-derived plasmids and the production and purification of fusion proteins have been published previously (12). Briefly, the open-reading frame encoding amino acids 28 to 190 of GRA1 or amino acids 40 to 150 (the N-terminal hydrophilic region) of GRA6 was cloned into the pGEX-3X or pGEX-2T expression vector, respectively. Both peptides were expressed in *Escherichia coli* BL21 as glutathione S-transferase (GST) fusion proteins and were purified by using glutathione agarose beads (Sigma, St Quentin, France). Wild-type GST protein was also produced and used as a control.

ELISA using both recombinant GRA1 and recombinant GRA6 Nt. The optimal concentration of recombinant antigens for the coating of flat-bottom microtiter plates (Greiner, Frickenhausen, Germany) and dilutions of serum, conjugate, and substrate were determined by using the checkerboard method. Different dilutions of each coating antigen and matched quantities of GST were investigated in a final volume of 100 μ l of carbonate-bicarbonate buffer (0.1 M; pH 9.6). Coated plates were hermetically sealed and stored at 4°C. Before use, they were washed three times with a standard washing solution (Enzygnost Toxoplasmosis IgG kit; Dade Behring) and blocked for 1 h at room temperature with 200 μ l of 5% skim milk solution in phosphate-buffered saline (pH 7.2). After five washes, 100 μ l of human serum diluted in peroxidase sample buffer (Enzygnost Toxoplasmosis IgG kit; Dade Behring) was added to each well and incubated at 37°C for 3 h. After a wash, each well was incubated for 1 h at 37°C with 100 μ l of anti-human IgG rabbit Fab' conjugated with peroxidase. After five washes, peroxidase activity was detected in the dark by use of tetramethylene benzidine dihydrochloride (Dade Behring). The reaction was stopped after 10 min by addition of 0.5 N sulfuric acid solution (Dade Behring). Absorbance was measured at 450 nm with a multiscan photometer (Thermolabsystems, Cergy Pontoise, France). Each sample was tested in triplicate on recombinant proteins, and the mean optical density (OD) was calculated on wild-type GST protein. The final result for each serum sample was calculated by subtracting the mean OD obtained for the control, GST, from the mean OD obtained for the recombinant fusion protein. A sample was considered positive if its final reading was higher than the mean of the negative samples plus 3 standard deviations (SD). Both the intra- and the interassay variability for each antigen were assessed by testing one positive and one negative serum sample 54 times in the same test and in triplicate in 16 different tests.

Statistical analyses. Differences between the acute and chronic profiles for GRA1 and GRA6 Nt were tested for significance by using the Mann-Whitney U test. A *P* value of <0.05 was considered statistically significant. Sensitivity and specificity were computed by using the time elapsed since infection (less than 3 months or more than 12 months) as the "gold standard."

RESULTS

Setting up the conditions for ELISA. The checkerboard assay performed with both the GRA1 and GRA6 Nt recombinant proteins determined the optimal working dilutions of 2 μ g/ml for GRA1 and 1.5 μ g/ml for GRA6 Nt. Serum samples were diluted 1:50 for the GRA1 ELISA and 1:75 for the GRA6 Nt ELISA. Dilutions used for the conjugate and for the substrate were 1:41 and 1:11, respectively. These experimental conditions yielded the greatest difference in ODs between positive and negative samples. The intra- and interassay reproducibilities were 2.9 and 8.8% for GRA1 and 4 and 8.3% for GRA6 Nt, respectively. The cutoff values were determined by calculating the mean of ODs observed for 100 negative serum samples plus 3 SD; cutoffs were 0.152 for GRA1 and 0.083 for GRA6 Nt. The same conditions were used for the GST ELISA, and an OD of 1.2 (mean plus 3 SD), calculated from results for the 231 samples included in the study, was chosen as the threshold. Two samples that scored above this value were considered ineligible for the study.

Reactivity of *T. gondii* antibodies to the GRA1 recombinant protein. Figure 1 shows the results of the ELISA performed on three groups of patients by using the GRA1 fusion protein. The specificity of this test was 98%, because two negative serum samples showed ODs above the cutoff. There was a significant difference in OD between groups 1 and 2 (*P* < 0.001). Interestingly, only 11 of 32 serum samples in group 1 were positive, whereas 36 of 46 scored positive in group 2, yielding sensitivities of 34 and 78.2%, respectively, with an overall value of 60%. The predictive value of a positive test score was 23% for acute infection and 76.5% for chronic disease.

Reactivity of *T. gondii* antibodies to the GRA6 recombinant protein. Results of the ELISA based on recombinant GRA6 Nt are presented on Fig. 2. A specificity of 98%, similar to that observed in the GRA1 test, was calculated. An important overlap in OD within the two positive groups was observed, with a nonsignificant difference in mean OD between the two groups (*P* = 0.88). The sensitivity of this ELISA was 83% for group 1 and 84% for group 2, with an overall value of 83%. The positive predictive value of the test was 41.5% for acute toxoplasmosis and 58% for chronic disease.

Combination of GRA1 and GRA6 Nt ELISAs. The possibility of improving the sensitivity of the test was addressed by combining the results obtained from the GRA6 Nt ELISA with those from the GRA1 ELISA (Table 1). Among the 31 samples found to be negative by the GRA1 ELISA, 22 were positive by the GRA6 Nt ELISA, corresponding to an improved sensitivity compared with GRA1 alone. Conversely, combining the samples that were positive with GRA6 and GRA1 resulted in a gain of six true positives, all originating from group 2, yielding a sensitivity of 91% and a specificity of 100%.

Since GRA1 seemed to react preferentially with serum samples from group 2, we investigated whether combining positive GRA1 and negative GRA6 Nt results could be a good serological marker to differentiate between diagnoses of acute and chronic toxoplasmosis. Such a combination, applied to group 2, yielded a sensitivity of 13% (6 samples out of 46) and a specificity of 100%, since none of the samples presenting an acute profile scored positive with this combination (data not shown).

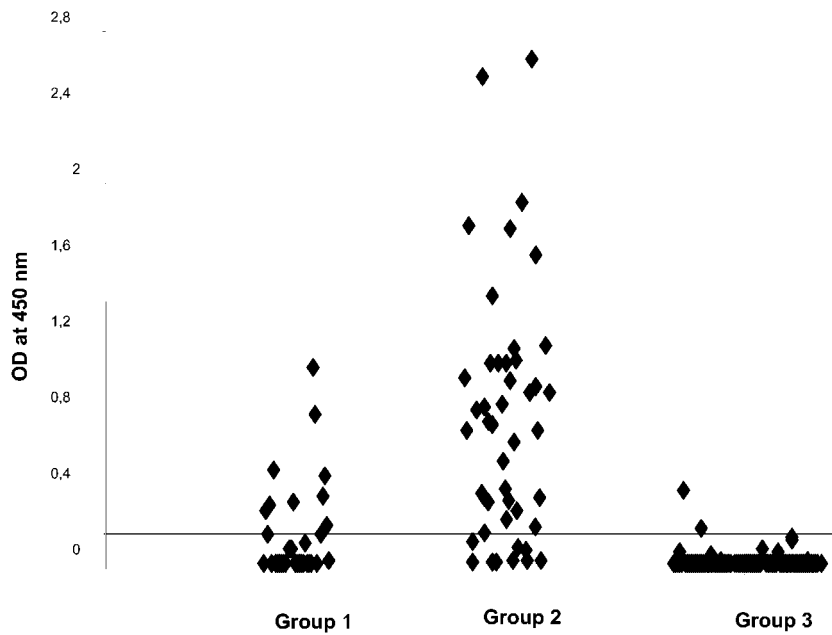


FIG. 1. GRA1-IgG ELISA prepared with serum samples from three groups of pregnant women. Group 1 comprises 32 serum samples from patients with acute infection; group 2 comprises 46 serum samples from patients with chronic infection; and group 3 includes 100 serum samples that tested negative by a standard diagnostic ELISA. Horizontal line indicates the cutoff value. The mean ODs for groups 1 and 2 are significantly different ($P < 0.0001$).

In addition, application of the GRA6-positive, GRA1-negative combination to group 1 yielded a sensitivity of 50% (16 samples out of 32). Moreover, the GRA6/GRA1 titer ratios of groups 1 and 2 did not allow a distinction between acute and chronic profiles (data not shown).

Follow-up of seroconverting women. For each of the 10 patients, a negative sample was drawn and the time that

elapsed between the taking of this sample and the first positive result was recorded. Additional serum samples were tested at various intervals. These samples covered at least the 2-month period that followed acute infection. The different serological profiles observed could be divided into three categories (Fig. 3). In four cases, the sample became positive simultaneously by routine ELISA and the anti-GRA1 and anti-GRA6 Nt ELISAs

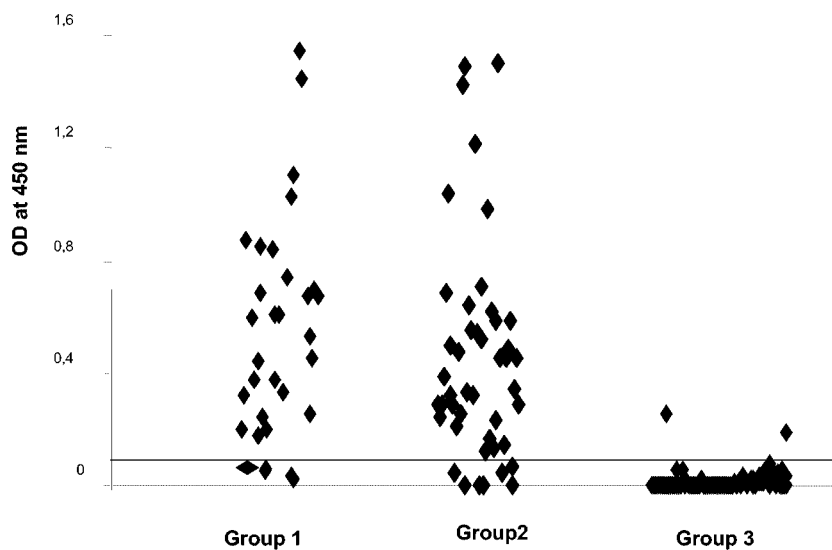


FIG. 2. GRA6 Nt-IgG ELISA prepared with serum samples from three groups of pregnant women. Group 1 includes 32 serum samples from patients with acute infection; group 2 corresponds to 46 serum samples from patients with chronic infection; and group 3 comprises 100 negative-control serum samples. Horizontal line indicates the cutoff value. The mean ODs for groups 1 and 2 are not significantly different ($P = 0.88$).

TABLE 1. Sensitivity improvement by combining results obtained with the GRA1 and GRA6 Nt-IgG ELISAs

Combination	No. with the indicated result ^a in groups 1 and 2 (n = 78)	Sensitivity (%)
GRA1-positive	47	60
GRA1-negative and GRA6-positive	22	28
Total		88
GRA6-positive	65	83.3
GRA6-negative and GRA1-positive	6	7.7
Total		91

^a Results from testing of 78 positive sera. Group 1, acute infection; group 2, chronic infection.

within 4 weeks after the last negative sample was drawn. (Fig. 3A). For such patients, neither GRA1 nor GRA6 Nt could distinguish between acute and chronic infections. In three cases, discordant profiles were observed: the anti-GRA6 Nt ELISA gave a positive score even before the routine ELISA, whereas GRA1 serology remained negative as late as 7 weeks after the first sample scored positive by routine ELISA (Fig. 3B). In the last category, both the GRA1 and the GRA6 Nt ELISA remained negative as long as 16 weeks after the first positive result by routine ELISA (Fig. 3C).

DISCUSSION

The aim of this study was to determine whether specific anti-GRA1 and/or anti-GRA6 Nt IgG could be a useful serological marker for differentiating between acute (acquired less than 3 months earlier) and chronic (acquired more than 1 year earlier) *Toxoplasma* infections in pregnant women. Since specific antibodies to human GST have been reported (20), a GST-IgG ELISA was systematically included as a control for each sample. Only 0.9% of serum samples scored above the threshold value. This percentage of reactivity with GST is lower than those reported previously (20, 22). Anti-GRA1 antibodies were detected in 78% of pregnant women with chronic infections (Fig. 1). The significant difference in mean OD between the two groups of patients suggests that anti-GRA1 antibodies could be serological markers of chronic infection, as reported previously (4). Nevertheless, 34% of women with acute infections scored positive for this antigen. This lack of sensitivity and specificity prevents the use of this test for the diagnosis of chronic infections. GRA6 Nt showed the same range of ODs for the two patient subgroups and did not seem efficient in distinguishing between acute and chronic infections (Fig. 2).

The combination of a positive GRA1 test with a negative GRA6 Nt test did not improve the sensitivity of the ELISA, whereas specificity reached a value of 100%. Combination of positive results obtained with either recombinant antigen reached a sensitivity of 91% for the detection of specific *T. gondii* antibodies irrespective of the date of contamination. The OD ratio obtained with the two antigens did not improve the accuracy of the test.

Follow-up of 10 women who seroconverted during pregnancy demonstrated the heterogeneity of the immune responses induced by these antigens (Fig. 3). The follow-up data clearly showed that the kinetics of the appearance of IgG

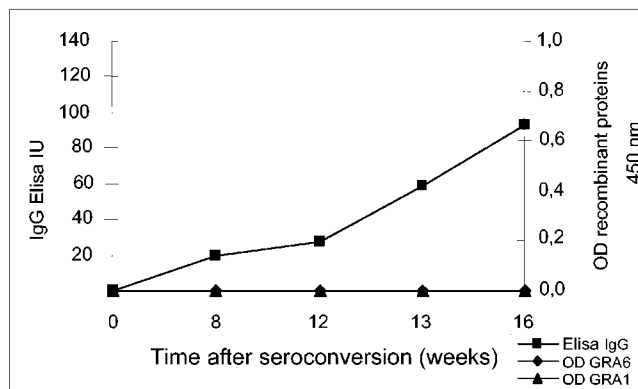
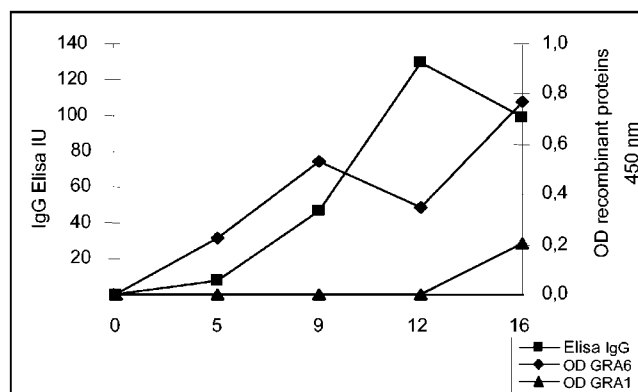
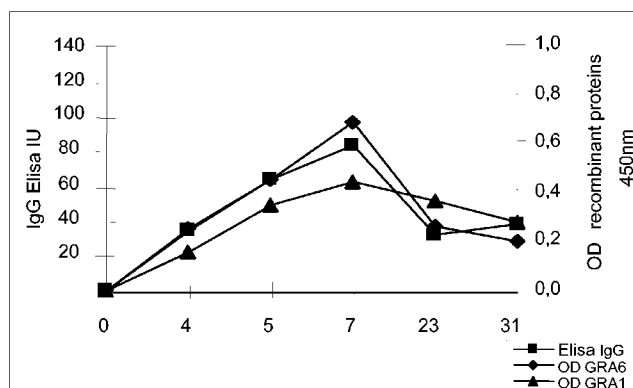


FIG. 3. Evolution of GRA1- and GRA6 Nt-specific IgG detected by ELISA compared with the evolution of IgG reacting with a parasite lysate (commercial kit) and expressed in international units. Follow-up of samples sequentially drawn from 10 pregnant women after seroconversion (represented by time zero). (A) Representative pattern of four cases: GRA6 Nt IgG and GRA1 IgG patterns correlated with the IgG profile obtained by routine ELISA. (B) Representative pattern of three cases: samples remained persistently negative by GRA1 ELISA. (C) Representative pattern of three cases: samples remained negative by both the GRA1 and GRA6 Nt ELISAs.

directed against each of these recombinant proteins is highly variable, preventing the use of GRA1 and GRA6 Nt as reliable markers of the stage of infection. Moreover, the negative patterns found in three patients raised the problem of antibody specificity for strain type. It has recently been demonstrated that peptides specific to one of the three main types of *T. gondii* strains could elicit a specific immune response, with no cross-

reactivity with the corresponding peptides derived from another type of strain (11).

The low overall sensitivity observed with GRA1 has already been reported by Lecordier et al. (12), although Beghetto et al. (2) showed that a recombinant GRA1 antigen fragment (residues 172 to 186) reacts strongly with serum samples from *T. gondii*-infected pregnant women. Lecordier et al. (12), using the same GRA6 Nt recombinant protein and testing 100 positive serum samples, reported a sensitivity of 96% compared with the value of 83% that we observed. Whether this difference is due to the size of the sampling remains to be investigated. They also observed that GRA1 may complement GRA6 Nt to improve sensitivity.

Several authors have reported attempts to distinguish between acute and chronic infections, but without conclusive results. Li et al. (13) reported that 85.3% of patients with acute toxoplasmosis reacted with a 35-kDa recombinant protein, whereas only 8% of patients with chronic infections gave positive results. Tenter et al. (22), combining two recombinant polypeptides termed H4/GST and H11/GST, found that 68% of patients with patterns of acute infection reacted positively with one or both antigens. Conversely, only 14% of those with chronic infections recognized one or both antigens. Using a recombinant GRA6 polypeptide, Redlich and Müller (20) reported 86% sensitivity and 99.6% specificity in the detection of acute toxoplasmosis. The main difficulty in comparing these results is that there is no consensus on the definition of either acute or chronic infection (21). Since the majority of *Toxoplasma* infections are sub-clinical, classification of serum samples into different groups sometimes relies only on the presence or absence of specific IgM. Such a classification is made problematic by the facts that the duration of IgM shows considerable variability among patients and that IgM can persist during the chronic phase (15). Additional *Toxoplasma* tests, such as the avidity assay (6) or the differential agglutination assay (7), could improve the accuracy of determination of the date of the infection, but these tests are limited by the heterogeneity of the immune response. French regulations, which offer non-immune pregnant women the possibility of being tested every month during pregnancy, enabled us to detect and define precisely the date of seroconversion. We investigated serum samples from women who had been infected less than 3 months and more than 1 year previously. A majority of pregnant women have their first routine *Toxoplasma* test during the first trimester of pregnancy. In such cases, therefore, it is critical to determine accurately whether infection occurred before or after the beginning of gestation. In contrast, determining that infection has occurred more than 1 year previously is important when samples are taken late in pregnancy.

In conclusion, GRA1 and GRA6 Nt ELISAs cannot accurately differentiate between acute and chronic toxoplasmosis. Currently, no accurate tool allows such differentiation, and the best way to rule out a postconception infection is to draw a sample as early as possible in pregnancy. The investigation of stage-specific antigens may be rewarding. Since the transformation from tachyzoite (the invasive stage) to bradyzoite within the organism is a rapid phenomenon with respect to antibody secretion, an overlap of immunological responses to

the two parasitic stages is likely to occur. More interestingly, at the immunological level, the avidity of antibodies toward stage-specific antigens could be a useful tool. One such study has already yielded promising results (3). Although recombinant proteins seem to be very promising for the diagnosis of toxoplasmosis, their performance, compared with that of the *Toxoplasma* lysates currently used in commercially available kits, needs to be improved, and the use of a cocktail of recombinant antigens may be advantageous. It has recently been demonstrated that combining peptides increases sensitivity in comparison with that of tests using the same antigens separately (1, 10). Moreover, extensive screening of recombinant antigen panels that are representative of the genetic diversity of *Toxoplasma* strains should be undertaken.

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